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Purification and Antiserum Preparation of Maize Dwarf Mosaic Virus[1]

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Abstract : A high purity of maize dwarf mosaic virus strain B (MDMV-B) with sufficient yield was obtained by isopycnic centrifugation in cesium sulphate. The virus was infectious and nonaggregated after the purification process. Ultraviolet absorption spectrum of the purified virus showed a typical curve of nucleroprotein with a maximum at 259 and a minimum at 249 nm. The yield of purified virus was 70—80μg per g of tissue. Antiserum to MDMV-B was produced by intramuscular injection of the purified virus to a rabbit. The highest titer of the antiserum obtained 7 weeks after the first injection was 1/8 in SDS-immunodiffusion test, and 1/2048 in precipitin ring test. The completely negative reactions of MDMV-B antiserum to healthy plant extract indicated the efficiency of this method for the purification of MDMV-B. A spur was produced between antigens of an isolate of sugarcane mosaic virus (SCMV) and MDMV-B against antiserum of MDMV-B in SDS-immunodiffusion test. Using the antiserum prepared in this study, we could easily distinguish MDMV-B from strains of SCMV by either SDS-immunodiffusion test or ELISA.

INTRODUCTION

Maize dwarf mosaic (MDM) was first noted as a new disease on corn in Ohio in 1962[10]. The disease was identified to be caused by a maize dwarf mosaic virus (MDMV)[28] or a strain of sugarcane mosaic virus (SCMV)[18] in 1965. Previously, sugarcane mosaic virus strain D (SCMV-D), the most common strain of SCMV on Badila cane, was reported to respond to corn mosaic in Taiwan[23]. However, the occurrence of maize dwarf mosaic virus strain B (MDMV-B) on corn has been reported by Deng[4] recently.

To our knowledge, one reason for the uncertainty in taxonomy of MDMV or SCMV strain was the lack of effective antisera[1,2,14,19,21,27]. Shepherd [18] was the first to purify these viruses, but the titers of antisera prepared by him were 1/256 for SCMV and 1/128 for MDMV in microprecipitin test. To purify those viruses and to prepare

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antisera, various methods were developed [1,2,7,8,9,11,12,14,15,17,19,22,26]. It was possible that the limited yield or the impurity of the purified virus resulted in the low titers or low specificities of antisera. In order to obtain a sufficient purified virus to produce a higher titer of antiserum, a procedure suitable for the purification of MDMV-B was developed in this experiment. This paper presents the results on the purification and antiserum preparation of MDMV-B. Part of the results was published elsewhere [5].

MATERIALS AND METHODS

Source of viruses: The virus for the purification in this study was originally obtained from a naturally infected corn plant in the experimental field of Taiwan Agricultural Research Institute. It was identified as maize dwarf mosaic virus strain B (MDMV-B) on the basis of failing to infect johnsongrass (*Sorghum halepense*) and sugarcane CP 31-294 [4]. Strains of MDMV-B, from infected corn plant in Chiayi, SCMV-B and SCMV-D [7], kindly provided by C. T. Cheng at Taiwan Sugar Research Institute, and S-1 isolate of SCMV, an unidentified strain from infected sugarcane CO 281, were used to compare and test the antiserum of MDMV-B. All virus strains/isolates were maintained on a susceptible corn cultivar Tainan No. 5 by mechanical inoculation in greenhouse.

Purification: Leaf tissues of MDMV-B infected corn plants grown in greenhouse were harvested 2—3 weeks after inoculation and then the midribs were removed. About 200g of leaf tissue was cut into 1cm long and homogenized in a prechilled cold Waring blendor with 300ml of 0.01M phosphate buffer, pH 7.0, containing 0.3% ascorbic acid, 0.01M sodium diethyldithiocarbamate, and 0.3% 2-mercaptoethanol. The homogenate was strained through two layers of cheesecloth, then clarified by adding chloroform with 1ml per 3g leaf tissue. The emulsion was broken by centrifugation at 8,000 rpm for 15min in a Hitachi RPR 12—2 rotor. The supernatant was collected and centrifuged at 30,000 rpm for 90min in a Hitachi RP 50—2 rotor. The resulting pellets were resuspended in 0.05 M borate containing 0.01M Na-EDTA, pH 8.2, and stirred overnight at 4°C. The virus suspension was centrifuged at 8,000 rpm (Hitachi RPR-16 rotor) for 15 min to remove the host components. The supernatant was the partially purified virus.

For further purification, cesium sulphate was added to the virus suspension to make the final concentration of 30 % (w/w), and run at 38,000 rpm for 22—24hr ($w^2t = 1.28 \times 10^{12}$ rad^2/sec) at 6°C in a Hitachi RP-65T rotor. The viral zone was collected from the tube by a small pippet after centrifugation. The purified virus was diluted with equal volume of 0.05M phosphate buffer. Low speed centrifugation was generally proceeded to remove the contaminants. Finally, the virus was dialyzed against the same buffer at 4°C overnight, and concentrated by centrifugation at 30,000 rpm for 90min.

Ultraviolet-absorption spectrum of the purified virus was measured in a dual-beam spectrophotometer (Hitachi 220S). Yield of the virus was estimated by assuming the extinction coefficient of MDMV-B to be 2.4/cm for 0.1% solution at 260nm [20].

Electron microscopy and infectivity test: In some cases, the purified preparations,

after negative staining, were examined with Hitachi H-300 electron microscope. In addition, the purified virus was made with serial dilutions in 0.01M phosphate buffer, and inoculated to 'Tainan No. 5' corn plants for infectivity test.

Production and testing of antiserum: Antiserum to MDMV-B was prepared by intramuscular injection of the purified virus into a rabbit weekly. Initially, 1mg of purified virus in 1ml of phosphate buffer was emulsified with 1ml of Freund's complete adjuvant and then injected. The following 3 injections, 2mg of the purified virus for each , were emulsified with incomplete adjuvant. The rabbit was started to bleed one week after the last injection. All antisera were perserved in the presence of 0.01% sodium azide prior to freezing.

The titers of antisera collected from different times were determined by SDS-immunodiffusion test[16], then the highest one was further checked by precipitin ring test[24]. In SDS-immunodiffusion test, fresh corn leaves infected with MDMV-B were ground in water, then added 3% sodium dodecyl sulfate (SDS). The medium for gel diffusion test consisted of 0.8% Noble agar, 0.5% SDS, 1% sodium azide, and 2% bovine serum albumin (BSA, Sigma Co.)[16]. In precipitin ring test, the purified virus was layered on the tops of serial antiserum dilutions in small tubes[24].

There were two MDMV-B isolates, one SCMV (S-1 isolate) and healthy corn plant for test of MDMV-B antiserum in SDS-immunodiffusion test. However, in addition to above virus isolates, strains of SCMV-B and SCMV-D were involved in enzyme-linked immunosorbent assay (ELISA). The purification and conjugation of immunoglobulin (IgG) with alkaline phosphatase were followed the procedures by Clark and Adams[3], and the double-antibody sandwich (DAS) method was adopted from Voller et al[25].

RESULTS

Purification: Our initial attempts to purify MDMV-B with methods previously described[2,17,18] did not result in satisfactory yield or purity. Because the broad (20mm wide) and ambiguous viral band formed in the tube was difficult to separate virus from plant materials by sucrose density gradient centrifugation. Purification of this virus by CsCl equilibrium centrifugation, a thin viral line was visible in the tube although all contaminants were well separated. Contrarily, the purified virus formed a dense band in Cs_2SO_4 solution after isopycnic centrifugation. In general, the viral band was located at 10—11.5mm below the meniscus and most of plant materials were remained at the top of the Cs_2SO_4 solution after centrifugation. It was easily to remove the purified virus from tubes (Fig. 1).

Ultraviolet absorption: The ultraviolet absorption spectrum of the purified virus as presented in Fig. 3, showed a curve typical for nucleroprotein with a maximum at 259 nm and a minimum at 249nm, but no shoulder at 290nm was observed. The value of A260/280 from several purified preprations ranged from 1.21 to 1.28. Based on the extinction coefficient of 2.4 for a 1-mg/ml solution[20], yield of the purified virus was estimated 70—80μg/g leaf tissue.

Electron microscopy and infectivity test: The purified MDMV-B as observed on

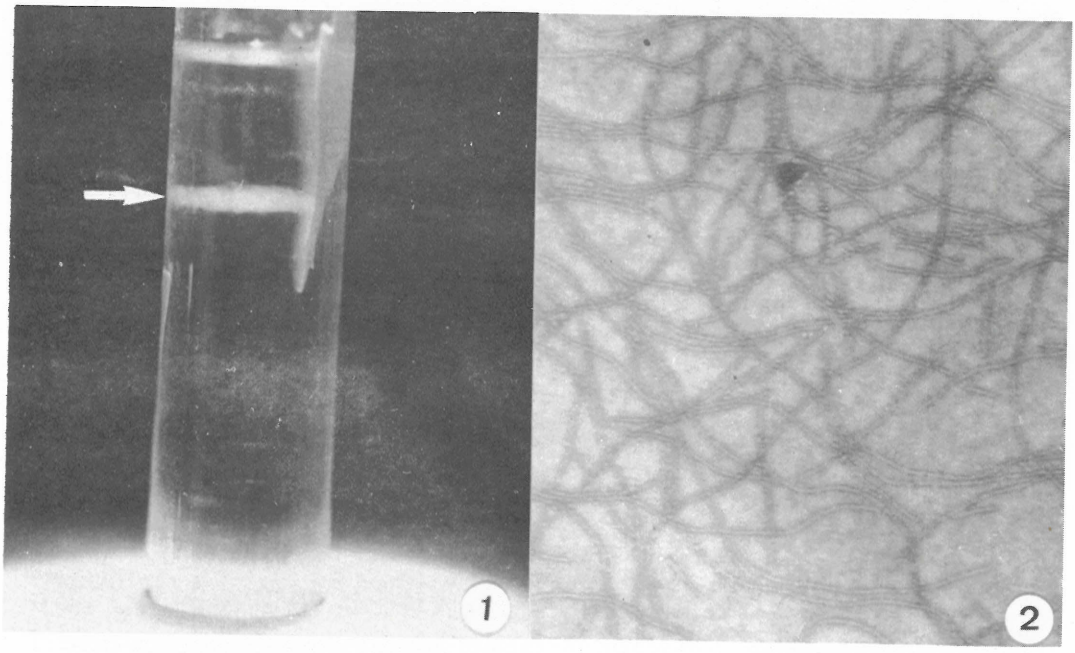

Fig. 1. A visible viral band (arrow indicated) formed 10—11.5mm below meniscus in the tube after centrifugation in cesium sulphate.

Fig. 2. Electron micrography of MDMV-B from purified preparation.

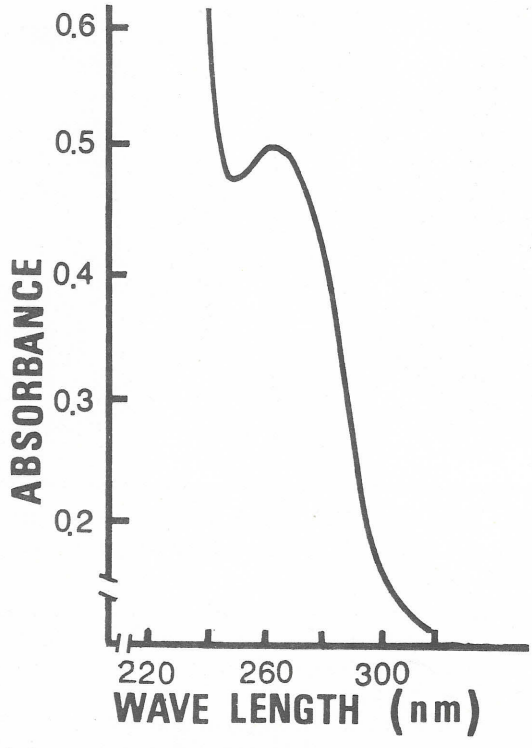

Fig. 3. Ultraviolet absorption spectrum of purified MDMV-B.

electron microscope showed numerous unaggregated filamentous particles (Fig. 2). For infectivity test, the purified preparation of MDMV-B was infectious to corn plants even it was diluted at 1/1280 (mg/ml) in 0.01M phosphate buffer.

Table 1. Reciprocal titers of MDMV-B antisera from six bleedings in SDS-immunodiffusion.

Bleeding of	Reciprocal titer with	
	MDMV-B	Healthy plant sap
1st	4	0
2nd	8	0
3rd	8	0
4th	8	0
5th	4	0
6th	2	0

Titer of antiserum: The immunized rabbit was bled six times totally. The titers of antisera for each bleeding were shown in Table 1 by SDS-immunodiffusion test. The highest one from second bleeding against homologous antigen was 1/8 in SDS-immunodiffusion (Fig. 4), and 1/2048 in precipitin ring test. No reactions were observed when all antisera from six bleedings were tested with healthy plant sap.

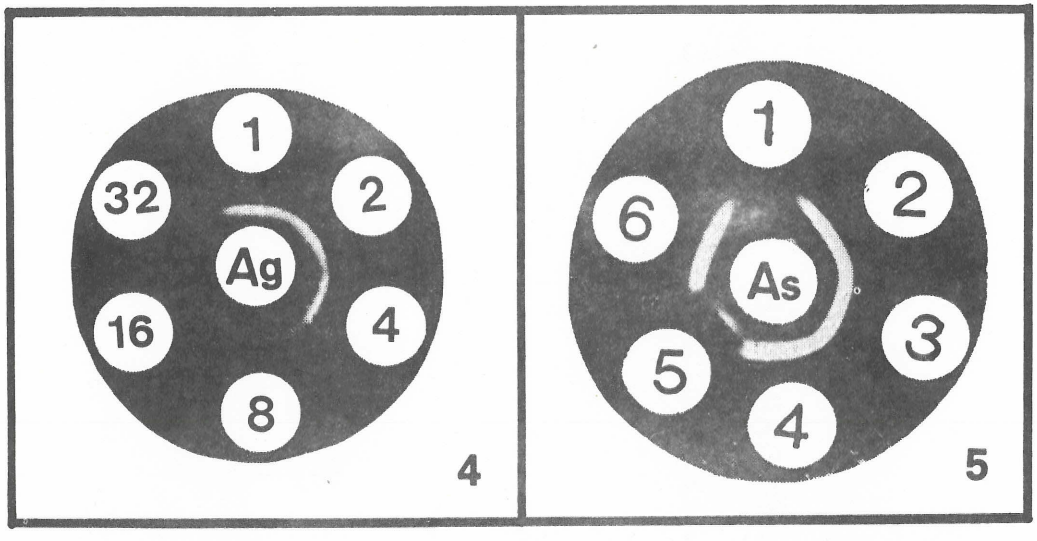

Fig. 4. Precipitation zones resulting from SDS-immunodiffusion test for checking titers of antiserum. The central well contained MDMV-B antigen and the peripheral wells contained serial dilutions of antiserum (numbers are dilution folds).

Fig. 5. Precipitin patterns formed in SDS-immunodiffusion test. The central well was charged with MDMV-B antiserum. The peripheral wells were charged with SDS-treated extracts from healthy corn 'TN 5' (1), MDMV-B infected corn 'TN 5' (2), another isolate of MDMV-B infected corn 'TN 5' (3), MDMV-B infected corn 'TN 351' (4), S-1 isolate of SCMV infected corn 'TN 5' (5), and MDMV-B infected sorghum 'Rio' (6). Spur formed between homologous and heterologous reactions and fused lines formed between homologous reactions.

Testing of antiserum: In SDS-immunodiffusion test, a comparison was made from the reacting patterns of MDMV-B (homologous) and SCMV (heterologous) forming in agar gel. It was clear that a dense precipitation band and a faint line were produced by homologous and heterologous antigens after incubation for 12 hours, respectively. As shown in Fig. 5, a spur was formed after a 2-day-period incubation between two precipitin lines.

In ELISA, the optimum working dilutions were 1μg/ml for coating IgG and 1/1000 for conjugated IgG, and the crude antigens were prepared at 1/10 (w/v) (Table 2). Table 3 showed the results of ELISA for MDMV-B and strains of SCMV. Usually the absorbance of MDMV-B was much higher than those obtained from strains of SCMV. In addition, the low value of the healthy plant sap reacted with MDMV-B antiserum made a clear background in ELISA test. Thus, we could easily distinguish MDMV-B from SCMV strains by this technique.

Table 2. Determination of appropriate working dilutions of reagents for ELISA

Coating IgG (μg/ml)	Dilution of conjugated IgG	Dilution of MDMV-B			Healthy (CK)
		1/10	1/100	1/1000	
2.00	1/500	1.456*	1.180	0.408	0.035
	1/1000	0.736	0.617	0.188	0.006
	1/2000	0.322	0.255	0.080	0.002
1.00	1/500	1.195	0.863	0.289	0.013
	1/1000	0.635	0.432	0.138	0.002
	1/2000	0.226	0.185	0.054	0.003
0.50	1/500	0.524	0.378	0.161	0.021
	1/1000	0.250	0.224	0.080	0.004
	1/200	0.108	0.098	0.033	0.001
0.25	1/200	0.027	0.022	0.000	0.004
	1/400	0.016	0.011	0.001	0.005

* The absorbance values (A_{405nm}) of the reacted substrate were read 30 minutes after incubation at 36°C.

Table 3. Comparison of homologous and heterologous antigens reacted with MDMV-B antibody by ELISA

Antigen	Absorbance at 405nm
MDMV-B	1.699
SCMV-B	0.592
SCMV-D	0.519
S-1 isolate	0.734
Healthy (CK)	0.011
PBS-Tween (blank)	0.000

1) Concentration of coating IgG was made at 1μg/ml, and conjugated IgG was at 1/1000 dilution. The antigens were prepared at 1/10 (w/v) in extraction buffer.

2) The absorbance value (A_{405nm}) of the reacted substrate was read after incubating 60 minutes at 36°C.

DISCUSSION

The problems encountered in purifying MDMV or SCMV are the low concentration in host, instability, and aggregation of the viruses during purification process, and contamination with host proteins in purified preparations [12,14]. The method developed in this study seemed satisfactory in purifying maize dwarf mosaic virus strain B.

Zea mays L. 'Golden Cross Bantam' is a very susceptible variety which has been widely used for propagating strains of MDMV and SCMV [1,12,14,18,20]. But the rapid decrease of extractable virions 21—28 days after inoculation in this host [13] may be the reason for low yield of the purified virus in previous studies. Since 'Tainan No. 5', a commercial cultivar, is as susceptible as 'Golden Cross Bantam' [4], and it grows more size of leaves in early growing stage, we chose it as the propagative host for our purification studies.

The additions of antioxidants and chelating agents are effective in preventing degradation and aggregation of virus during purification process [20]. The unbuffered solution of ascorbic acid, 2-mercaptoethanol, and DIECA had been applied to extract several SCMV and MDMV strains [2,6,10], and the yields of purified virus were much higher than those of virus extracted in 0.5M citrate buffer [19]. It is advantageous to keep pH near neutrality throughout the whole purification process, therefore, instead of water, we used 0.01M phosphate buffer for virus extraction. Chloroform has been routinely used for the clarification of corn tissue extract [20], but it reduced the viral infectivity if a higher concentration was applied in the purification of MDMV [8,12,14,19,20]. The low concentration of chloroform had been recommended by several workers [22,26], but we obtained a quite infectious purified virus without aggregation when chloroform concentration was added at 1ml per 3g tissue.

Pirone and Aznalone [15] emphasized that all SCMV would lose if a second high speed centrifugation was performed. In our experiment, we still recovered fair amounts

of MDMV-B after two cycles of differential centrifugation. The loss of virus was possibly due to the effects of different buffer systems and/or virus strains. Sucrose density gradient centrifugation had been used conventionally for further purification of MDMV and SCMV [6,12,14,15,18,22], but our results indicated that a broad viral band was formed after centrifugation, and lots of contaminants were collected together. Purification of MDMV and SCMV had been greatly improved by CsCl equilibrium centrifugation [6,8,9,17,26], but Snazell *et al.* [19] found that CsCl or sucrose rate-zonal centrifugation yielded no difference. In this study, the yield of purified virus from Cs_2SO_4 centrifugation was higher than that obtained from CsCl centrifugation indicating that the low pH of CsCl solution may affect the stability of virus during the equilibrium centrifugation, and reduced the yield of purified virus.

Ultraviolet absorption spectrum is a criterion for measuring purity of final sample [6,8,12,,14,17,22,26]. Pirone and Anzalone [15] could not obtain a typical spectrum of purified SCMV although little extraneous material was visible in the EM. Our initial trial showed that there was no typical UV-absorption curve when the virus was purified by sucrose density gradient centrifugation. However, the virus purified by Cs_2SO_4 centrifugation, the typical curve for nucleroprotein was obtained. The slight shoulder of UV absorption spectrum at 290nm or 288nm was not observed in our study although it was reported by many authors [8,12,14,26]. Anyway, our result agreed to the report of Sehgal and Jean [17]. The value of A260/280 ranged from 1.21 to 1.28 and the yield of purified virus about 70—80 μg/g leaf tissue as comparing to the other reports [2,6,8,9,11,12,14,17,21,26], it seems that we have improved the method in purification of MDMV-B.

Previously, microprecipitin test was used to serotype the strains of MDMV and SCMV [1,2,7,11,18,19,21,26,27], but MDMV-B, SCMV-B, or SCMV-D were serologically indistinguishable in those reports [11,21]. The MDMV-B antiserum as prepared in this experiment produced a dense preciptin line with its homologous antigen and formed spurred reactions with its heterologous antigens in SDS-immunodiffusion tests suggesting that this antiserum can be used to differentiate MDMV-B from SCMV strains. Details for comparison among strains of MDMV and SCMV, and field studies are in progress.

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玉米矮化嵌紋病毒之純化與抗血清製作[1]

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摘 要

利用等密度平衡離心法純化玉米矮化嵌紋病毒 B 型系統，以提高病毒純度及產量。純化所得之MDMV-B 仍具相當之感染力，且純化過程並無病毒聚集現象。以紫外光吸收光譜測定病毒純度，可得一標準的核蛋白吸收曲線，其最高值在259nm最低值在249nm。純化的病毒產量爲每克葉片有70—80微克。利用純化的MDMV-B 對白兎行肌肉注射以製作抗血清，其力價以瓊脂擴散反應測定最高爲1/8，但以沈澱環反應測定則爲 1/2048。此等抗血清完全不與健康的玉米株汁液反應，可見利用本方法已獲得良好純化效果。從甘蔗分離出來而在玉米上繁殖的一個甘蔗嵌紋病毒系統與MDMV-B 抗血清有反應，但是在瓊脂擴散反應試驗時形成刺狀沈澱帶，由此可以區分MDMV-B和本省之SCMV 系統。另由酵素結合抗體免疫分析的結果，MDMV-B、SCMV-B及SCMV-D對 MDMV-B 抗體反應的讀值，同樣可以確認其間的異同。

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